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Regulation of Hydrogen Sulfide Liberation in Wine-Producing *Saccharomyces cerevisiae* Strains by Assimilable Nitrogen

V. JIRANEK,[1]† P. LANGRIDGE,[2] AND P. A. HENSCHKE[1]*

The Australian Wine Research Institute, Glen Osmond,[1] and Department of Plant Science, Waite Agricultural Research Institute, The University of Adelaide,[2] South Australia 5064, Australia

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Saccharomyces cerevisiae **wine-producing yeast cultures grown under model winemaking conditions could be induced to liberate hydrogen sulfide (H_2S) by starvation for assimilable nitrogen. The amount of H_2S produced was dependent on the yeast strain, the sulfur precursor compound, the culture growth rate, and the activity of the sulfite reductase enzyme (EC 1.8.1.2) immediately before nitrogen depletion. Increased H_2S formation relative to its utilization by metabolism was not a consequence of a de novo synthesis of sulfite reductase. The greatest amount of H_2S was produced when nitrogen became depleted during the exponential phase of growth or during growth on amino acids capable of supporting short doubling times. Both sulfate and sulfite were able to act as substrates for the generation of H_2S in the absence of assimilable nitrogen; however, sulfate reduction was tightly regulated, leading to limited H_2S liberation, whereas sulfite reduction appeared to be uncontrolled. In addition to ammonium, most amino acids were able to suppress the liberation of excess H_2S when added as sole sources of nitrogen, particularly for one of the strains studied. Cysteine was the most notable exception, inducing the liberation of H_2S at levels exceeding that of the nitrogen-depleted control. Threonine and proline also proved to be poor substitutes for ammonium. These data suggest that any compound that can efficiently generate sulfide-binding nitrogenous precursors of organic sulfur compounds will prevent the liberation of excess H_2S.**

Several mechanisms have been proposed to explain the liberation or excessive production of hydrogen sulfide (H_2S) during the fermentation of grape juice to wine. Early research determined that elemental sulfur residues which are chemically reduced directly to H_2S were an important cause of this problem (1, 32, 38). Identification of the sources of these residues, specifically fungicides used on vines or sulfur candles used for cooperage sterilization, prompted changes in viticultural and winemaking practices to minimize the carryover of sulfur to the fermentation. Strict adherence to withholding periods when using fungicides containing colloidal sulfur has been especially important (44, 45). Despite the resulting large decline in their incidence, H_2S problems remain as one of the most common fermentation problems encountered by the wine industry.

Other proposed mechanisms for H_2S production largely focused on cysteine because of its potency when added directly to a yeast culture (11–13, 32, 47). Although cysteine-hydrolyzing enzymes which release H_2S have been described (2, 46), cysteine is not important to excessive H_2S production during winemaking because of its scarcity in grape juice (3, 18). Additional pools of cysteine have been proposed to be liberated from must proteins by a yeast extracellular proteolytic activity induced by nitrogen starvation (12, 13, 47). No suitable proteolytic or autolytic degradation of grape proteins and yeast cells has been demonstrated experimentally (14, 23, 30, 36, 42). Thus, while a link between nitrogen depletion and H_2S liberation continues to be reported (17, 27, 28, 40, 47), the direct involvement of cysteine has not been demonstrated.

Most recently, Stratford and Rose (40) reported that the liberation of H_2S arises primarily from a reduction of inorganic sulfur to H_2S in excess of cell requirements. Hydrogen sulfide is routinely formed in controlled amounts by this pathway (Fig. 1) as a prelude to the incorporation of sulfur into methionine and cysteine. This synthesis is necessary since grape juice typically cannot fulfill yeast sulfur requirements in the form of organic sulfur compounds. Ordinarily, feedback inhibition (4, 9, 16, 50) and gene repression by end products, especially methionine derivatives (5–7), ensures that the activity of the sulfate reduction sequence (SRS) is tightly regulated to match the metabolic demand for methionine and cysteine. A starvation for nitrogen could be expected to deplete the cell of these regulatory end products, resulting in a derepression of the structural genes of the SRS enzymes and hence an increased flux of sulfur through the pathway. A similar methionine shortage and hence overproduction of H_2S can develop from deficiencies of vitamins which act as cofactors to SRS enzymes (2, 12, 27), but the common use of vitamin supplements prevents this route of H_2S overproduction from being significant.

The question of whether such a nitrogen-dependent mechanism for H_2S production operates in the wine fermentation has not been clearly resolved. This is largely attributable to the use of media, typically grape juices, of ill-defined nitrogen and sulfur content and degree of aeration (12, 13, 32, 38). The latter is especially important in affecting nitrogen utilization (20), fermentation vigor, and hence H_2S stripping from the medium. Failure to clearly define the role of nitrogen in H_2S production in the wine fermentation may account for the continued reference in the literature to alternate mechanisms involving elemental sulfur and cysteine (33, 34) despite the above-cited reports showing them to be of no practical importance.

In this study, we confirm that the liberation of H_2S by wine-producing yeast strains under model winemaking conditions

* Corresponding author. Mailing address: The Australian Wine Research Institute, P. O. Box 197, Glen Osmond, SA 5064, Australia. Phone: 61 8 303 6600. Fax: 61 8 303 6601.

† Present address: Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213.

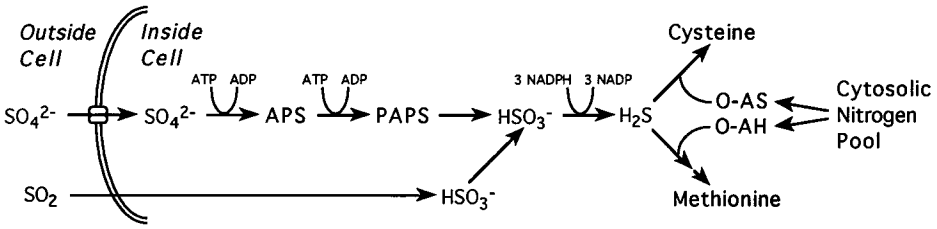

FIG. 1. SRS and sulfur amino acid biosynthesis. Abbreviations: APS, adenosyl 5′-phosphosulfate; PAPS, 3′-phosphoadenosyl 5′-phosphosulfate; O-AS, *O*-acetylserine; O-AH, *O*-acetylhomoserine.

occurs as a response to nitrogen starvation, is fueled by inorganic sulfur, and involves the sulfite reductase enzyme. The greater potency of sulfite as a precursor has been demonstrated, as has the varied efficiency of amino acids to suppress this production.

(Preliminary aspects of this work were presented at the International Symposium on Nitrogen in Grapes and Wine, 22 to 24 June 1991, Seattle, Wash.)

MATERIALS AND METHODS

Organisms and culture media. *Saccharomyces cerevisiae* strains AWRI 77 and AWRI 72 were obtained as freeze-dried cultures from the Australian Wine Research Institute culture collection. A chemically defined grape juice medium (GJM) approximating the composition of typical grape juice has been reported previously (18). To facilitate monitoring of the concentration of residual assimilable nitrogen, the described mix of amino acids was replaced by NH_4Cl. Sodium metabisulfite was added to 260 μM. Grape juice starter medium differed from GJM in containing glucose at 555 mM, ergosterol to 25 μM, and monooleate (Tween 80) to 0.05% (vol/vol).

Experimental cultures. A single yeast colony was seeded into 25 ml of MYPG medium (malt extract, 3 g/liter; yeast extract, 3 g/liter; peptone, 5 g/liter; glucose, 10 g/liter) contained in a loosely capped conical flask and incubated overnight with shaking (180 oscillations per min) at 25°C. Organisms were subcultured to 2×10^6 cells per ml into 25 ml of grape juice starter medium in 250-ml baffled Erlenmeyer flasks plugged with cotton wool and incubated to early stationary phase (ca. 24 h). This culture served as the inoculum for fermentation trials and was used at 5×10^6 cells per ml.

Fermentation trials were conducted in 250-ml screw-cap conical flasks fitted with a sidearm port sealed with a rubber septum for aseptic and anaerobic sampling and supplementation. Customized glass tubing (250 mm; 8-mm outside diameter, 3-mm inside diameter) was attached to the flask neck by a compression cap and O-ring. The outlet end was curved 180° and drawn down to accept 5 mm of Teflon tubing to which a 100-μl micropipette was attached. The micropipette was immersed in 10 ml of trapping solution to create a fermentation lock and a trap for H_2S which emerged with gases exiting the fermentation flask. Trapping solution (described below) was contained in a 30-ml bottle clipped to the flask neck and protected from extended light exposure to prevent photooxidation of cadmium sulfide (1).

The inoculated medium (150 ml) was poured into sterile flasks, fermentation locks and H_2S traps were fitted, and an anaerobic headspace was established by the introduction of 100 volumes of oxygen-stripped (Oxypurge N; Alltech) nitrogen delivered via an 18-gauge needle inserted through the flask septum. Throughout the fermentation, flasks were sparged with purified nitrogen at a rate of 1 ml/ml of medium per min. The gas was delivered to the bottom of the flask through an 18-gauge needle of appropriate length. Flasks were incubated with shaking (180 oscillations per min) at 25°C.

Growth was monitored spectrophotometrically at 650 nm (Varian model 635 spectrophotometer). Culture biomass was interpolated from a calibration curve relating dry weight to culture absorbance. Fermentation progress was monitored by refractive index of the cell-free fermentation medium and related to glucose concentration by standard curve. Fermentations were complete at glucose concentrations of below 2.5 g/liter as estimated by using $CuSO_4$-NaOH tablets (Clinitest; Ames). Residual ammonium and glucose were quantified by using enzymatic test kits (Boehringer Mannheim). Protein quantitation was achieved by a modification of the Lowry method (39).

Cycloheximide supplementation experiments. Strain AWRI 77 was inoculated into GJM containing 8.3 mM ammonium and 260 μM sulfite. Approximately 1 h before nitrogen was predicted to become depleted, cultures were either unsupplemented or supplemented with ammonium to 11.1 mM either alone or together with cycloheximide to 35.5 mM. Culture samples were harvested at intervals over 8 h, cell extracts were prepared, and their sulfite reductase activity was determined.

Assay of sulfite reductase activity. The NADPH-dependent sulfite reductase activity of cell extracts was determined by measuring the formation of H_2S from sodium sulfite according to a modification of an existing method (31). Culture samples were cooled on ice and subsequently maintained below 2°C. The equivalent of 200 mg (dry weight) of cells (ca. 10^{10} cells) was collected by centrifugation, washed twice with equal volumes of 0.25 M phosphate buffer (pH 7.3) containing 1 mM EGTA, and resuspended in 5 ml of the same clarified (0.45-μm-pore-size membrane) buffer containing 20% (vol/vol) glycerol. The inclusion of glycerol from this point onward was required since in our hands sulfite reductase activity was found to be cold sensitive (21). After the addition of 10 g of glass beads (diameter, 0.45 to 0.5 mm; Braun), cells were shaken in a cell homogenizer (Braun model MSK) with CO_2 cooling at 2,000 rpm until >99% disruption was achieved (determined by microscopic examination). Glass beads were removed by filtration through a glass sinter funnel, and cellular debris was removed from the filtrate by centrifugation at $50,000 \times g$ for 30 min. Dialysis was performed for two periods of 75 min, each against 1,000 volumes of fresh resuspension buffer.

The reaction mixture was prepared in the resuspension buffer and contained glucose-6-phosphate (1.7 mM), $MgCl_2$ (1 mM), Na_2SO_3 (0.1 mM), NADP (0.1 mM), and glucose-6-phosphate dehydrogenase (166 U/liter). A stock solution of Na_2SO_3 was prepared fresh before use.

For each sample to be assayed, 3 ml of reaction mixture was dispensed in triplicate into 10-ml disposable tubes. Cell extract (routinely 400 μl) was added to the tubes, and the total volume was adjusted to 4 ml with resuspension buffer. Tubes were quickly stoppered with septa (Suba-seal no. 29) and gently inverted several times before being incubated at 30°C for 1 h. Working-strength amine reagent (300 μl; see below) was immediately injected into the tubes, and the contents were mixed and incubated at ambient temperature for 1 h. Samples were routinely centrifuged at $12,000 \times g$ for 5 min to remove any precipitate which may have formed. The extinction at 672 nm of clarified samples was measured. Blanks and standards were identical to samples in all respects except that cell extracts were boiled for 3 min or substituted for with known amounts of H_2S. H_2S formation was calculated from a standard curve prepared from the latter.

Enzyme activity was expressed against culture dry weight rather than cellular protein content to facilitate comparison with H_2S production rates from fermentations. More importantly, however, this measure of yeast biomass proved more stable across the extremes of nitrogen availability encountered in this investigation (19).

Analysis. Quantitation of H_2S liberated from cultures was achieved by an optimization of previously reported methods (1, 15, 35). H_2S was collected from fermentation gases in 10-ml aliquots of cadmium hydroxide trapping solution prepared from $3CdSO_4 \cdot 8H_2O$ (4.3 g/liter) and NaOH (0.6 g/liter). H_2S traps were changed periodically. New micropipettes were fitted at this time, since the incumbent micropipettes frequently contained precipitated CdS and so were retained with the corresponding trap. A stock amine reagent of 0.75 M *N*,*N*-diethyl-*p*-phenylenediamine · HCl in 11.3 M H_2SO_4 was stored in the dark at 4°C. Working-strength reagent was prepared by diluting the stock reagent 20-fold in 9 M H_2SO_4 and adding $FeCl_3$ solution (60%, wt/wt) at the rate of 1:12 immediately prior to use. Screw-cap bottles (30 ml) containing neat or diluted trapping solution (10 ml) were dosed with 325 μl of working-strength amine reagent, closed immediately, and mixed vigorously for 30 s. Sample absorbance (672 nm) was measured after 1 h of incubation at ambient temperature. The H_2S content of samples was calculated from absorbances of known amounts of sulfide in the range of 0 to 12.1 μg.

Measurement of the accumulation and fate of [^{35}S]sulfate. The protocol for the measurement of the rate of [^{35}S]sulfate accumulation by yeast cells has been described previously (41). Radioactive tracer ($Na_2{}^{35}SO_4$; Amersham) was added to the fermentation medium (4 μCi/mmol, specific activity) before inoculation. Two-milliliter samples were withdrawn at intervals throughout the fermentation. Cells were rapidly harvested from 1-ml aliquots of the sample by vacuum filtration through nitrocellulose membrane filters (0.45-μm pore size, 25-mm diameter) and washed with 12 ml of ice-cold citrate buffer (20 mM, pH 3.5) containing 5 mM unlabelled sulfate. Filters were then placed into 1.5-ml microcentrifuge tubes and dosed with 1.1 ml of scintillation fluid (ASC II; Amersham), and the

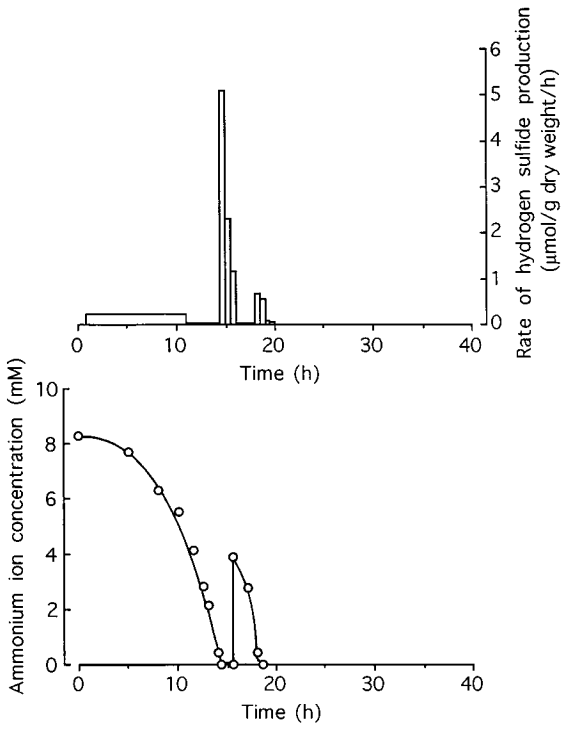

FIG. 2. Ammonium concentration (○) and H_2S production (histogram) by strain AWRI 77 grown in GJM containing sulfite (260 μM). NH_4Cl was added at 16.5 h to a concentration of 4.2 mM.

radioactivity was quantified in a liquid scintillation spectrometer (Beckman model 3600).

H_2S formation from [^{35}S]sulfate was determined by quantitation of radioactivity collected in H_2S traps. Before colorimetric quantitation of H_2S, a 250-μl sample of trapping solution was transferred into a microcentrifuge tube and quantified as described above by liquid scintillation.

RESULTS

Influence of assimilable nitrogen on hydrogen sulfide liberation. Two *S. cerevisiae* strains were chosen for this study: AWRI 77, a popular commercial wine-producing yeast strain which is a moderate producer of H_2S, and AWRI 72, which is a high-H_2S-producing yeast strain when tested on BiGGY agar (Difco) (21). The temporal relationship between a depletion of assimilable nitrogen and the evolution of H_2S by these strains was studied in a model GJM. Qualitative findings were similar for the two strains; hence, only data for AWRI 77 are shown (Fig. 2). For both strains, low rates of H_2S liberation in the presence of nitrogen were increased severalfold following nitrogen depletion from the medium and then restored through ammonium ion supplementation. Low rates were sustained until the supplementary ammonium was depleted, whereafter increased rates of H_2S liberation reappeared. Strain AWRI 72 differed from strain AWRI 77 in producing greater amounts of H_2S overall and continuing to liberate H_2S (ca. 0.2 μmol/g [dry weight]/h) following ammonium supplementation.

Individual or pairs of amino acids were tested to determine how they compared with ammonium in suppressing the liberation of H_2S induced by a depletion of ammonium. Yeast cells were first cultivated in GJM containing ammonium and sulfite. Then, 1 h before the ammonium was predicted to become exhausted, aliquots of the fermentation medium were supplemented with the nitrogen compound(s) under test. The amount of H_2S liberated by these cultures was taken to indicate the ability of the nitrogen compound to substitute for ammonium in suppressing the excessive production of H_2S. For AWRI 77, most of the amino acids tested demonstrated a suppressive capacity similar to that of ammonium (Table 1). By comparison, AWRI 72 was responsive to a smaller number of amino acids; nine amino acids permitted the liberation of H_2S in amounts similar to those for the unsupplemented control. In common with AWRI 77, proline, threonine, and cysteine were without effect on the suppression of H_2S. Indeed, supplementation of fermentations with cysteine, alone or in combination with methionine, led to increased rates of H_2S liberation compared with the unsupplemented control for both strains. This increase was about 10-fold for AWRI 77 and 25- to 35-fold for AWRI 72.

H_2S liberation by AWRI 77 following the depletion of nitrogen was dependent upon the growth phase of the culture. Maximum rates and extents of liberation were observed when depletion of nitrogen occurred during the exponential phase of growth (Fig. 3). Conversely, when depletion of nitrogen occurred during the stationary phase of growth, H_2S liberation was short-lived and at a reduced rate.

Precursor for hydrogen sulfide production. The high rates of H_2S liberation which were induced by a depletion of assimilable nitrogen occurred only from media containing sulfite in addition to sulfate. As shown in Fig. 4, when sulfate was the sole sulfur source (i.e., prior to ca. 15 h), the depletion of nitrogen from the medium (at ca. 12 h) induced only a short-lived production of H_2S at a relatively low rate of ca. 2.5 μmol/g (dry weight)/h. Although H_2S formation from sulfate had ceased by the 15-h time point, this culture could still produce H_2S in large amounts, as shown after sulfite addition to the medium. The nearly complete absence of tracer in H_2S collected from fermentation gases after sulfite supplementation confirmed that sulfate reduction continued to be limited even during this second peak of H_2S-liberating activity. The low rate of H_2S formation from sulfate strongly contrasts with rates of label incorporation from [^{35}S]sulfate into yeast biomass. In the presence of nitrogen, label incorporation typically reflected the increase in biomass and attained a peak rate of ca. 42.5 μmol/g (dry weight)/h immediately prior to nitrogen depletion (data not shown).

Role of sulfite reductase. The importance of sulfite as a precursor for the production of H_2S strongly implicates sulfite reductase in this process. Given the involvement of this enzyme in the biosynthesis of the sulfur-amino acids, its activity could be expected to reflect the metabolic or growth-related demand for these amino acids. In turn, the rate of H_2S liberation upon nitrogen depletion could be predicted to be proportional to the culture growth rates which immediately preceded the depletion of nitrogen from the medium. This supposition was supported indirectly by several experiments. Taken as a measure of culture growth rate, the specific rate of nitrogen utilization during nitrogen sufficiency was positively correlated with both total H_2S liberation ($r = 0.961$) and the mean rate of H_2S liberation over 12 h ($r = 0.832$) after nitrogen depletion (Fig. 3). Alternatively, when culture growth rates were manipulated by using amino acid nitrogen sources of differing efficiencies (Table 2), a highly significant inverse correlation ($r = 0.994$) was observed between the level of H_2S liberation upon nitrogen depletion and culture doubling times.

Quantitation of the NADPH-dependent sulfite reductase activity of extracts of nitrogen-sufficient cultures (Fig. 5) showed that the rates of H_2S production observed in these assays resembled those of H_2S liberation by cultures which became nitrogen limited at comparable stages of growth (Fig. 3). Maximal rates of H_2S production were similar in the two

TABLE 1. Suppression of H_2S production by amino acids and ammonium[a]

Strain AWRI 77		Strain AWRI 72	
Nitrogen source added	Relative H_2S production[b]	Nitrogen source added	Relative H_2S production
None	61.82 ± 17.96	None	158.41 ± 58.64
NH_3	1.00 ± 0.29	NH_3	2.87 ± 0.71
Ser	0.71 ± 0.07	Gln	6.27 ± 3.36
Gln	0.91 ± 0.16	Asn	7.50 ± 3.57
Ala	1.00 ± 0.14	Ile	10.09 ± 3.09
Asp	1.09 ± 0.48	Asp	11.00 ± 8.55
Arg	1.16 ± 0.43	Phe	12.48 ± 0.64
Asn	1.55 ± 0.27	Arg	17.57 ± 13.48
Glu	1.57 ± 1.14	Ser	25.23 ± 4.55
γ-Aba	1.91 ± 1.02	His	27.05 ± 4.55
Ile	1.93 ± 0.25	γ-Aba	29.77 ± 18.71
Phe	1.98 ± 0.52	Thr	64.32 ± 37.27
Trp	6.66 ± 4.68	Trp	76.59 ± 31.82
His	7.71 ± 11.75	Lys	122.27 ± 49.55
Lys	7.77 ± 3.68	Pro	122.96 ± 6.64
Gly	8.91 ± 6.68	Glu	123.64 ± 46.59
Met	13.09 ± 11.23	Met + Thr	123.64 ± 2.36
Val	14.98 ± 7.07	Ala	139.77 ± 40.68
Pro	59.32 ± 26.82	Met	150.46 ± 7.27
Met + Thr	132.27 ± 10.68	Gly	165.68 ± 1.82
Thr	134.32 ± 99.32	Val	194.55 ± 44.09
Met + Cys	395.46 ± 54.77	Cys	3,945.46 ± 309.09
Cys	590.91 ± 393.18	Met + Cys	5,775.00 ± 681.82

[a] Fermentations were initiated in GJM (4.5 liter) containing sulfite (260 μM) and ammonium (8.3 mM). One hour prior to ammonium depletion, aliquots (180 ml) were anaerobically transferred into fermentation flasks (250 ml), supplemented with amino acids or ammonium to a total equivalent of 14.3 mM nitrogen, and monitored for H_2S production over 6 h. All samples except the unsupplemented control were confirmed to contain residual assimilable nitrogen.

[b] Expressed as a proportion of the value for the ammonium-supplemented cuture of strain AWRI 77, which produced 13 nmol/liter. Values are means of at least two determinations ± standard error of the mean and are not adjusted for growth differences.

experiments (5.6 versus 7.8 μmol/g [dry weight]/h). In both cases, these peaks of activity occurred during the exponential phase of growth. Furthermore, when H_2S liberation was induced by nitrogen starvation during the stationary phase of the culture, the short-lived and reduced rate of H_2S liberation was reflected by a corresponding low sulfite reductase activity of the nitrogen-sufficient culture at that time.

These findings suggest that the excessive production of H_2S which is observed following nitrogen depletion is a consequence of the action of an existing sulfite reductase activity rather that a newly synthesized one resulting from a derepression of the structural gene encoding the enzyme. To test this notion further, NADPH-dependent sulfite reductase activity and H_2S liberation were determined for the same culture across two cycles of nitrogen sufficiency and starvation. As shown in Table 3, sulfite reductase activity was largely independent of the nitrogen status of the culture. By comparison, H_2S liberation ranged between trace in the presence of nitrogen and high during nitrogen starvation. Furthermore, this high rate of H_2S production corresponded to that predicted by sulfite reductase assays.

Further evidence opposing a de novo synthesis of sulfite reductase comes from a similar experiment in which the rates of decay of enzyme activity were compared in nitrogen-limited cultures which were either unsupplemented or supplemented with nitrogen either alone or together with cycloheximide (see Materials and Methods). In the nitrogen-supplemented culture, sulfite reductase activity was essentially stable at 11.5 μmol of H_2S per g [dry weight] per h over the course of the experiment. By comparison, both the unsupplemented culture and the cycloheximide-supplemented culture displayed a sulfite reductase activity which was stable only for ca. 90 min following nitrogen depletion and cycloheximide addition. En-

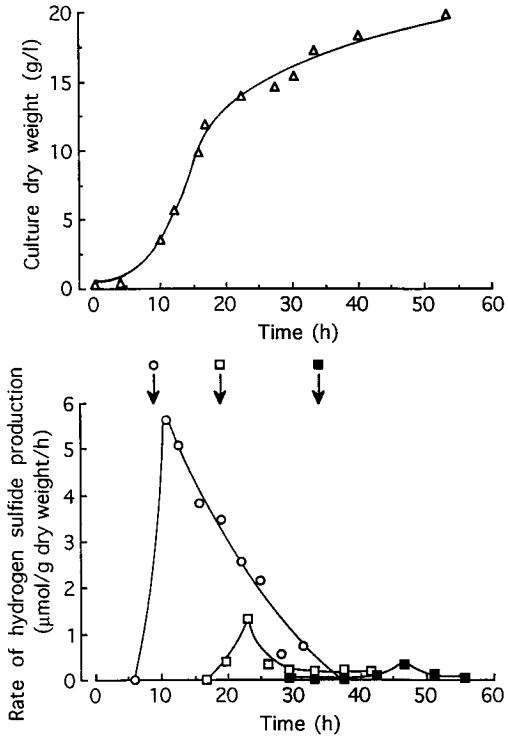

FIG. 3. Effect on H_2S production by cultures of strain AWRI 77 of ammonium depletion at progressively later stages of growth. Cultures were grown in GJM which initially contained a limiting ammonium concentration of 3.3 (○), 16.6 (□), or 24.9 (■) mM. A control culture which contained an excess ammonium concentration of 33.3 mM (△) did not produce H_2S (data not shown) and provided data for the growth curve (top). Sulfite was added to 132 μM at 1 h prior to the point (labelled arrows) of ammonium depletion, and H_2S production was monitored for a further 24 h.

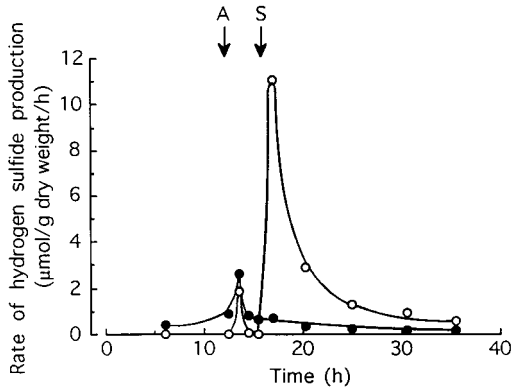

FIG. 4. Total H_2S production (○) and production from [^{35}S]sulfate (●) by cultures of strain AWRI 77. Fermentations were conducted in GJM containing 8.3 mM NH_4Cl and [^{35}S]sulfate (5 mM; 4 μCi/mmol, specific activity). Sulfite was added to 260 μM at the time indicated (S) following the point of ammonium depletion (A).

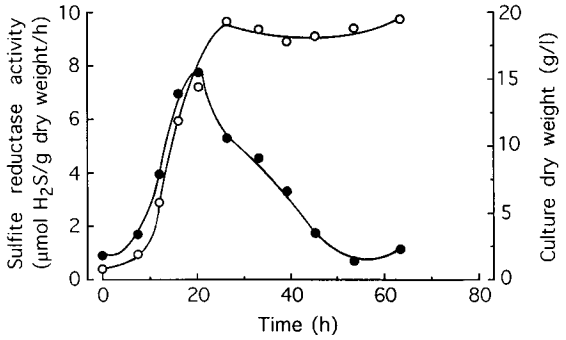

FIG. 5. Sulfite reductase activity during the growth of strain AWRI 77. Fermentations were conducted at 25°C in synthetic GJM containing ammonium (33.3 mM) and sulfite (260 μM). Biomass formation (○) and NADPH-linked sulfite reductase activity (●) were determined at regular intervals throughout fermentation.

zyme activity subsequently decayed in both cultures with a half-life of approximately 4 h.

DISCUSSION

Typically, yeast cells produce only enough H_2S to meet biosynthetic requirements. An excess of H_2S relative to metabolic requirements can presumably arise through either its increased formation or its reduced consumption by metabolism. Any H_2S formed beyond these requirements diffuses from the cell into the fermentation medium. The continuous sparging of experimental cultures and a selective trapping of H_2S allows this excess H_2S to be quantified.

In this report, we have identified factors which influence the extent of H_2S liberation by wine-producing yeast strains. By confirming earlier findings for the cider fermentation (40) in wine-producing yeast cultures, we have demonstrated that assimilable nitrogen is also a key factor regulating H_2S liberation in enology. The observation that such liberation is suppressed by ammonium supplementation of the medium agrees with the empirical finding of the fermentation industries that assimilable nitrogen, typically ammonium or urea, frequently remedies an H_2S problem. This study was not, however, limited to the effects of ammonium alone. Numerous amino acids were tested, and many were found to be as effective as ammonium in suppressing H_2S liberation, particularly by strain AWRI 77. This finding suggests that the amino acids largely act as nitrogen sources feeding into transamination reactions as shown for brewing yeast strains (22). Differences in the suppressive capacity of individual amino acids may arise from the ease with which they enter the cytosolic nitrogen pool and/or supply cysteine and methionine biosynthesis which consumes cellular H_2S. Indeed, the most potent amino acid suppressants of H_2S liberation are typically those which support high growth rates (8), i.e., serine, glutamine, ammonium, aspartate, arginine, and asparagine, or amino acids which act as direct precursors for *O*-acetylserine or *O*-acetylhomoserine synthesis, i.e., serine and aspartate.

Amino acids with a regulatory role in the SRS and/or sulfur amino acid biosynthesis or those which contain sulfur are the major exceptions. For example, threonine inhibits the biosynthesis of homoserine, a precursor of methionine (49). Thus, irrespective of the availability of an average nitrogen source, insufficient regulatory methionine derivatives are formed. The result is not only a derepression of the SRS (5–7), and thus increased H_2S formation, but also a reduced incorporation of H_2S into methionine. By comparison, cysteine induces the largest liberation of H_2S despite regulating the SRS, either itself (10) or through an interconversion with methionine (7, 25). It is likely cysteine is being enzymatically catabolized to ammonium, pyruvate, and H_2S directly (2, 46).

TABLE 2. Influence of nitrogen source on culture doubling time and H_2S production by strain AWRI 77[a]

Nitrogen source	Doubling time (min)	H_2S production[b]	
		Maximum rate (μmol/g [dry wt]/h)	Total[c] (μmol)
NH_4^+	324	2.04	16.40
Arg	348	1.35	11.03
Phe	408	1.38	3.49
Tyr	450	1.37	0.94
Arg, Phe, Tyr	384	0.99	7.72

[a] Fermentations were conducted in GJM containing 520 μM sulfite. Assimilable nitrogen was provided to a total nitrogen concentration of 7.5 mM by a single nitrogen compound or an equal ratio of three amino acids. Cultures were monitored for a total of 49.5 h, which was 36 h beyond nitrogen depletion in the ammonium-grown culture.

[b] Values are means of two determinations.

[c] Sum of H_2S determinations for samples collected throughout the experiment.

TABLE 3. Effect of nitrogen status on H_2S production and sulfite reductase activity[a]

Nitrogen present	Sulfite reductase activity (μmol/g [dry wt]/h)	H_2S production rate (μmol/g [dry wt]/h)
+	8.3	ND[b]
−	12.7	12.5
+	13.5	ND
−	11.8[c]	11.7

[a] Strain AWRI 77 was grown in GJM containing ammonium (8.3 mM) and sodium metabisulfite (260 μM). At a point 45 min after the initial supply of ammonium in the medium was depleted, additional ammonium was added to 4.2 mM. A second addition was made in the same manner once ammonium was again exhausted. H_2S was collected over 30 min approximately 1 h before the initial supply of ammonium became depleted and thereafter over 30-min periods immediately after the culture entered the described state of nitrogen availability. Culture samples for the determination of sulfite reductase activity were harvested at the end of each H_2S collection period.

[b] ND, not detectable.

[c] Interpolated from bracketing determinations.

Cysteine-hydrolyzing enzymes do not otherwise appear to be important in the H_2S formation seen in this study or in winemaking for several reasons. The medium used here was not prepared with cysteine, and grape juices typically contain only traces of cysteine (3, 18). An autolytic or proteolytic activity would have little impact on H_2S production since protein, from which to release cysteine, was absent from GJM. Similarly, cellular cytosolic pools of cysteine are minute (48). In fact, even a complete catabolism of all cellular sulfur-amino acids would yield only ca. 23% of the H_2S observed here. The frequently cited cysteine-degradative route to H_2S production is therefore not supported.

It is interesting to compare the responses of AWRI 77 and AWRI 72 in the nitrogen supplementation experiments described above. Most notably, methionine repressed H_2S liberation by AWRI 77 more effectively than that by AWRI 72 (Table 1). The unresponsiveness of AWRI 72 to methionine regulation suggests that either this strain has a highly active methionine-cysteine interconversion and degradation pathway which releases H_2S directly, or else it fails to either synthesize or respond to the appropriate regulatory methionine derivative(s). Since H_2S liberation by AWRI 72 is not completely suppressed by assimilable nitrogen, the latter two options appear more likely. Furthermore, a strain partly responsive to methionine regulation would not be expected to be induced to H_2S production following the addition of threonine, an inhibitor of methionine synthesis. Accordingly, the threonine-supplemented cultures represent the one instance in which more H_2S is liberated by AWRI 77 than the high-H_2S-producing AWRI 72 (Table 1). A detailed investigation of the sulfite reductase activity of this strain under various degrees of nitrogen availability should clarify this issue.

Sulfate and sulfite are the predominant sulfur sources in wine fermentation (3). Aside from the amino acid supplementation experiments described above, sulfate and sulfite were the only sources of sulfur used in this study and thus are the only possible precursors for H_2S production. The use of radioactive tracer demonstrated that both compounds were able to fuel H_2S production, though to quite different degrees (Fig. 4). The superiority of sulfite as a precursor for this production, even when present at 1/19 the concentration of sulfate, agrees with previous reports of sulfite reduction to the complete exclusion of sulfate when the two precursors were present together (40). The present findings stress that sulfate is nevertheless an important precursor compound in fermentations using this wine-producing yeast strain and that the simple omission of sulfite will not guarantee protection from H_2S formation. At any rate, the existence of high-sulfite-producing strains is well known.

The lesser efficiency of sulfate as a precursor for H_2S production following nitrogen starvation indicates the existence of a mechanism limiting the reduction of this compound under these conditions. This mechanism appears to target sulfate transport or a step of the SRS upstream of sulfite reductase since sulfite reduction is not affected (Fig. 4). Feedback inhibition of SRS enzymes (4, 9, 10, 16, 50) by SRS intermediates, including H_2S, is likely to be rendered ineffective by the ready diffusion of H_2S from the cell. Consequently, the futile reduction of sulfate to H_2S during nitrogen starvation is proposed to be prevented by a blockage of sulfate transport. This blockage may result from an inability of nitrogen-starved cells to maintain high rates of transport protein turnover (24, 37). H_2S that is formed from sulfate following nitrogen starvation may merely represent the finite pool of sulfate in the cytosol following transport shutdown. In accordance with this view, the observed H_2S liberation of 1.8 μmol/g (dry weight) (Fig. 4) would require 170 μg of sulfate, compared with estimates of cytosolic sulfate pools of between 30 and 120 μg/g (dry weight) (26). The existence of a sulfate-specific control mechanism incapable of limiting the reduction of exogenous sulfite can be rationalized in an evolutionary sense by realizing that the sulfite-supplemented winemaking fermentation is quite distinct from the natural environment of yeasts in which they are rarely exposed to sulfite.

The ready reduction of exogenously supplied sulfite strongly implicates a central role for sulfite reductase, the terminal enzyme of the SRS and the only reported route for sulfite reduction to H_2S by *S. cerevisiae* (29, 31, 50). The ability of sulfite reductase to accommodate the observed maximum rate of H_2S liberation of 13.5 μmol/g (dry weight)/h is apparent since [^{35}S]sulfur was accumulated into yeast biomass and hence reduced by this enzyme at a peak rate of 42.5 μmol/g (dry weight)/h. The difference between these rates may be indicative of energetic limitations following nitrogen depletion. Given that sulfite is reduced in a seemingly uncontrolled manner during nitrogen starvation, a principal determinant of the amount of H_2S liberated will be the level of sulfite reductase activity of a culture prior to nitrogen starvation. Factors influencing this level will be the strains genetically determined level of sulfite reductase activity or its propensity for H_2S production (21, 43, 51) and the demand for sulfur amino acids before nitrogen starvation. Supporting this view is the finding that indirect measures of this demand, specifically growth and nitrogen utilization rates, were correlated with H_2S liberation after nitrogen starvation. Similarly, the greatest liberation of H_2S developed in response to a depletion of nitrogen during the high-growth exponential phase (Fig. 3). Analogous observations have been reported for strain TC8 under laboratory conditions (40).

Possible mechanisms by which H_2S production exceeds its rate of incorporation into biomass are numerous. By the two foremost mechanisms, nitrogen starvation causes a depletion of regulatory methionine derivatives and so a derepression of SRS enzymes and hence an increased formation of H_2S. Alternatively, H_2S is no longer sequestered into sulfur amino acids because of a shortage of the appropriate nitrogenous precursors. In view of the limited ability of sulfate to fuel H_2S production following nitrogen depletion, derepression of SRS enzymes other than sulfite reductase would be of little importance. In any case, significant de novo synthesis of sulfite reductase is not supported by the relative stability of enzyme activity across extremes of nitrogen availability (Table 3). Moreover, when produced, H_2S liberation occurred at rates equal to the existing level of sulfite reductase activity. Another consideration is whether a nitrogen-starved cell is able to achieve a de novo synthesis of sulfite reductase let alone within the short time frame of the H_2S response. The finding that cycloheximide produced a response similar to that produced by nitrogen starvation for either sulfite reductase activity or H_2S liberation rates (21, 40) refutes this notion.

Instead, the mechanism of excessive H_2S production most likely to be in operation during a nitrogen deficiency in enology appears to be the ongoing reduction of sulfite by a preexisting sulfite reductase activity, despite the depletion of the nitrogenous precursors of methionine and cysteine synthesis which ordinarily condense with H_2S. Uncombined H_2S readily diffuses from the cell to enter the fermentation, where it becomes apparent. The key substrate for this process is the energetically preferred exogenous sulfite moiety which freely diffuses and, in fact, can accumulate in the cell (41). The rate of production of H_2S is suggested to be primarily determined by the level of sulfite reductase activity immediately prior to nitrogen starva-

tion, which in turn is a reflection of the prior demand for methionine and cysteine. Measurements of existing levels of sulfite reductase activity generally exceeded and so accounted for the observed rates of H_2S liberation. Thus, in the absence of elemental sulfur residues or vitamin deficiencies, a depletion of assimilable nitrogen and in turn a diminished incorporation of reduced inorganic sulfur is the primary cause of excessive H_2S formation by wine-producing yeast strains under wine-making conditions. Future studies will focus on confirming sulfate transport as the control point for sulfate reduction during nitrogen starvation and understanding the process by which sulfite reduction ultimately declines.

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